DIAZEPAM RECEPTOR: SPECIFIC BINDING OF [^{3}H]DIAZEPAM AND [^{3}H]FLUNITRAZEPAM TO RAT BRAIN SUBFRACTIONS

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1. Introduction

Evidence is beginning to accumulate that specific molecules bind the benzodiazepines in a receptor-related manner [1–4]. However, to date, no studies have demonstrated to which cellular structures the diazepam was bound. Morphologic confirmation is essential when one is dealing with the central nervous system, since 'standard' fractionation procedures for one area in the brain give varying results in other regions of the brain [5]. We report here that the fraction of rat forebrain containing synaptic junctions binds specifically [^{3}H]diazepam; furthermore, we show that [^{3}H]flunitrazepam binds to the benzodiazepine 'receptors' with more than 10-times greater affinity in the described system than [^{3}H]diazepam and hence may be a useful tool for further investigation.

2. Materials and methods

[*methyl*-^{3}H]Diazepam and [*methyl*-^{3}H]flunitrazepam were obtained from New England Nuclear Co., Boston, MA and each had a spec. act. 40 Ci/mmol. Pure unlabeled diazepam was a gift of Roche Laboratories, Nutley, NJ. It was determined that the forebrain specifically bound the most [^{3}H]diazepam of all areas of the brain [4]. Forebrain sections of 150–200 g Sprague-Dawley or Holtzman rats were homogenized in 10 vol. 0.32 M sucrose, and the homogenate centrifuged at 1000 × *g* for 10 min. Fractionation procedures of the brain sections were described [6,8]. The principal contents of the respective fractions are shown in table 1 and fig.1.

To determine the specific binding, 500 μl samples of the fractions, resuspended in 50 mM Tris buffer, in 0.32 M sucrose were exposed to either 5 nM [^{3}H]diazepam or 5 nM [^{3}H]diazepam plus 3 μM unlabeled diazepam. The assay tubes were then incubated at 37°C for 15 min with shaking, followed by equilibration for 30 min in an ice bath. To collect the fraction binding the [^{3}H]diazepam, 10 ml ice-cold 50 mM Tris buffer, pH 7.5, was added to each incubation tube and the contents poured over Whatman GF/A glass fiber filters with suction. The filters were washed free of unbound diazepam with an additional 10 ml Tris buffer and placed in 5 ml Bray's solution. [^{3}H]Diazepam was measured in a Nuclear Chicago scintillation counter. Specifically-bound [^{3}H]diazepam, as given herein, was the activity in the assay tube incubated with only the [^{3}H]diazepam minus the activity in the appropriate assay tube with the [^{3}H]diazepam and the large excess of unlabeled diazepam, as is the convention in the determination of specific binding. All assays contained 5 nM [^{3}H]diazepam since 16% available diazepam was bound at this concentration [4]. This allowed for greater of lesser binding to be measured in the various experiments, while still maintaining a 600 M excess in the nonspecific assay tubes.

Because of its high binding activity [4] (table 1), the B fraction was used for the kinetic studies with the [^{3}H]flunitrazepam. Aliquots, 500 μl, of the B fraction (4 mg protein/assay) were incubated with the [^{3}H]flunitrazepam or [^{3}H]flunitrazepam plus unlabeled

Table 1
Specific binding of [^{3}H]diazepam by various fractions of the total forebrain of the rat

Fraction or subfraction	Predominant morphologic content[c]	fmol/mg protein[b]
A fraction	Myelin	5 ± 0.9
B fraction[a]	'Purified synaptosomes', myelin and mitochondria	91 ± 7.0
C fraction	Mitochondria	9 ± 0.9
D subfraction of B	Large and small synaptic vesicles, membranes and junctions	38 ± 4.0
E subfraction of B	Myelin and membranes	66 ± 7.0
F subfraction of B	Myelin, large vesicles and junctions	47 ± 4.0
G subfraction of B[a]	Membranes and junctions	182 ± 27.0
H subfraction of B[a]	Synaptosomal membranes and junctions	392 ± 48.0
I subfraction of B	Mitochondria	48 ± 8.0

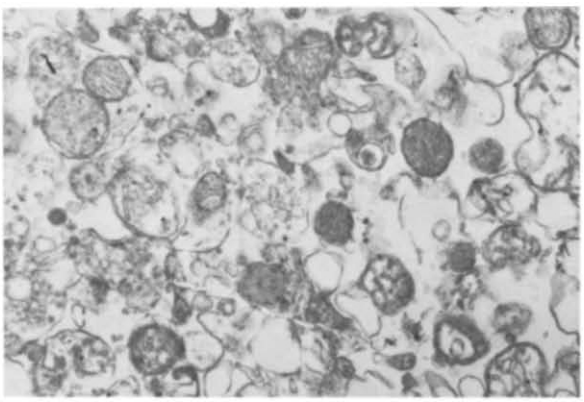
[a] Electron micrographs are shown in fig.1. Suspension of the various fractions were incubated with 5 nM [^{3}H]diazepam (see text) to determine specific binding
[b] Mean ± SD
[c] Description of the morphologic content was based on electron micrographs of each fraction or subfraction

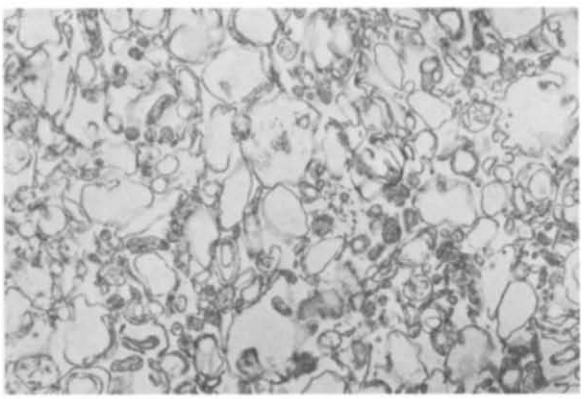
diazepam at concentrations ranging from 0.01–500 nM. Thus diazepam was used to determine non-specific binding and displacement. Electron microscopy was performed on each fraction as given in the legend to fig.1. Protein was measured as in [9].

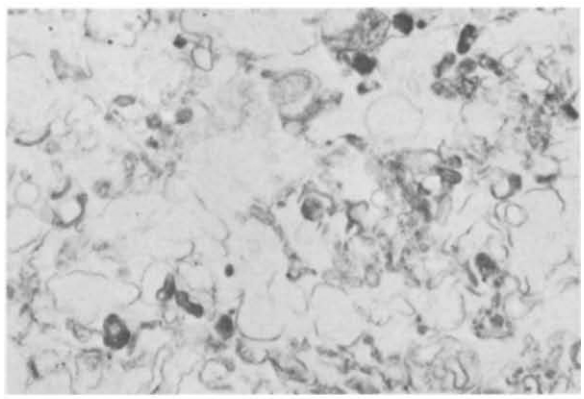
3. Results and discussion

It is evident that fraction H, containing the synaptosomal membranes and junctions, bound specifically the highest amount of [^{3}H]diazepam (table 1; fig.1, bottom). Fraction G, which also contains membranes and junctions, specifically bound the second highest amount of [^{3}H]diazepam (table 1; fig.2, middle). It should be noted that the mitochondria comparatively do not bind significant amounts of [^{3}H]diazepam. Thus the highest number of macromolecules specifically binding [^{3}H]diazepam are in the synaptosome membrane and junction fraction(s). Since all the fractions were radiolabeled prior to microscopy, light microscopic autoradiography was performed on 1 μm plastic-embedded sections. It is of interest that the only fraction activating photographic grains was fraction H. Thus the high affinity binding of the synaptosomal membranes and junctions was retained in the presence of 2% glutaraldehyde and subsequent alcoholic dehydration.

As shown in fig.2, [^{3}H]flunitrazepam bound to fraction B had app. K_d 1.1 nM. Since under identical conditions [4] [^{3}H]diazepam bound to fraction B with app. K_d 14.8 nM, it is obvious that [^{3}H]flunitrazepam has a greater affinity for the benzodiazepine receptor. Furthermore, kinetic analysis of fraction B showed the maximum number of binding sites with [^{3}H]diazepam to be 363 fmol/mg protein, with [^{3}H]flunitrazepam to be 171 fmol/mg protein and for 1:1 molar mixtures of [^{3}H]diazepam and [^{3}H]diazepam to be 368 fmol/mg protein. Thus it is obvious that the [^{3}H]flunitrazepam may be a more powerful probe for the benzodiazepine receptor.







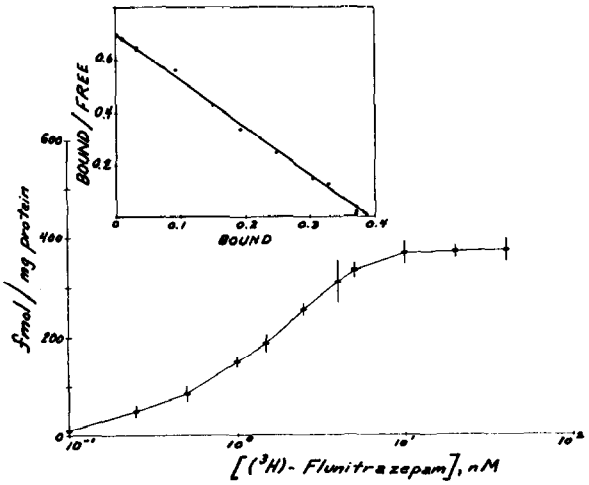


Fig.2. Binding of [^{3}H]flunitrazepam to purified synaptosomal (fraction B) macromolecules as a function of concentration of [^{3}H]flunitrazepam. Each assay was performed as given in the text and each point is the mean ± 1 SD. The insert is a Scatchard analysis of the data. Free and bound units are pmol/mg protein/500 μl. The fact that a straight line was obtained indicates that all of the binding sites have the same intrinsic binding constant (commonly referred to as one binding site) with affinity, app. K_d 1.1 nM. Also, maximal binding occurs at 371 fmol [^{3}H]flunitrazepam/mg purified forebrain nerve-ending protein (fraction B).

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Fig.1. Electron micrographs of thin sections from fractions B (top), G (middle) and H (bottom) of [^{3}H]diazepam. Fractions were fixed in 2% buffered glutaraldehyde, post-fixed in 1% OsO_4, dehydrated in ethanol, embedded in epoxy resin, sectioned and double-stained with lead citrate and uranyl acetate. All micrographs are 18 000 ×. Fraction B contains membranes, mitochondria, synaptic junctions and myelin. Subfraction G contains membranes, membrane vesicles and junctions. Subfraction H contains membranes, synaptic junctions and membrane vesicles.

References

[1] Squires, R. F. and Braestrup, C. (1977) Nature 266, 732–734.
[2] Mohler, H. and Okada, T. (1977) Life Sci. 20, 2101–2110.
[3] Braestrup, C. and Squires, R. F. (1977) Proc. Natl. Acad. Sci. USA 74, 3805–3809.
[4] Bosmann, H. B., Case, K. R. and DiStefano, P. (1977) FEBS Lett. 82, 368–372.
[5] McLaughlin, J., Case, K. R. and Bosmann, H. B. (1973) Biochem. J. 136, 919–926.
[6] Gray, E. G. and Whittaker, V. P. (1962) J. Anat. 36, 79–88.
[7] Bosmann, H. B. and Hemsworth, B. A. (1970) J. Biol. Chem. 245, 363–371.
[8] Bosmann, H. B. (1972) J. Biol. Chem. 247, 130–145.
[9] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.